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Gephyrin, a master regulator of neuronal function?

Shiva K. Tyagarajan and Jean-Marc Fritschy*

*Corresponding author:
Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH – 8057 Zurich, Switzerland
e-mail: fritschy@pharma.uzh.ch

Author's biographical sketches

Jean-Marc Fritschy trained as a neuromorphologist at the University of Lausanne (Switzerland) and at the Johns Hopkins University (Baltimore, MD). He is Faculty member at the University of Zurich (Switzerland) since 2000. His research focuses on the molecular and functional organizations of GABAergic synapses and their significance for brain development, adult neurogenesis, and pathophysiology of temporal lobe epilepsy.

Shiva Tyagarajan graduated as a molecular virologist at the Pennsylvania State University (USA) and later trained as a neurobiologist during his post-doctoral studies at the University of Zurich. He is currently a group leader and the research focus of his group is to elucidate molecular and cellular mechanisms that couple transcriptional and post-transcriptional programs to regulate GABAergic synaptic plasticity.

Abstract

The neurotransmitters GABA and glycine mediate fast synaptic inhibition by activating ligand-gated Cl^- channels, namely the $GABA_A$ and glycine receptors. Both types of receptors are anchored postsynaptically by gephyrin, which self-assembles into a scaffold and interacts with the cytoskeleton. Current research indicates that gephyrin postsynaptic clusters are dynamic assemblies that are held together and regulated by multiple protein–protein interactions. Moreover, posttranslational modifications of gephyrin regulate the formation and plasticity of GABAergic synapses, by altering the clustering properties of postsynaptic scaffolds and thereby the availability and function of receptors and other signalling molecules. Here, we discuss the formation and regulation of the gephyrin scaffold, its role in GABAergic and glycinergic synaptic function and the implications for the pathophysiology of brain disorders caused by abnormal inhibitory neurotransmission.

On-line summary

- Gephyrin is a multi-functional protein, responsible for Moco biosynthesis in all organisms, and for postsynaptic clustering of glycine receptors and GABAA receptors in vertebrate CNS
- Gephyrin forms a protein scaffold by self-assembly from trimeric complexes, which interacts with numerous, structurally different proteins, in order to form a high ordered signalling complex in glycinergic and GABAergic synapses
- Gephyrin function as a scaffolding protein is regulated by alternate mRNA splicing and by multiple post-transcriptional and posttranslational modifications, which only begin to be understood.
- Regulation of gephyrin scaffold by multiple signalling cascades modulate the formation and plasticity of GABAergic synapses, and thereby the strength of GABAergic transmission.
- Because signals impinging on gephyrin posttranslational modification are activated by excitatory neurotransmission and increased intracellular Ca2+ concentration, we discuss here the evidence that gephyrin scaffold forms an intracellular hub modulating synaptic homeostasis and excitatory-inhibitory balance.
- Abnormal GABAergic transmission during brain development, possibly brought about or at least linked to impaired gephyrin regulation, might have enduring structural and functional consequences in adult brain, and might contribute to the pathophysiology of major neurological and neuropsychiatric diseases. Here, these issues are discussed with a focus on their significance for the development of novel therapeutic approaches.

Introduction

At chemical synapses, the postsynaptic density (PSD) precisely faces the presynaptic active zone and contains a high concentration of ligand-gated ion channels. These cardinal features of synapses ensure that sharp, high-amplitude signals are generated following quantal neurotransmitter release. Moreover, they imply that sophisticated molecular mechanisms exist to enable the assembly of macromolecular complexes in the PSD and the selective clustering of the neurotransmitter receptors at appropriate subcellular sites.

Many aspects of postsynaptic receptor clustering and its functional importance remain poorly understood, although the complexity of this process is being increasingly appreciated. The scaffolding proteins involved in such clustering are often signalling molecules themselves or anchor other signalling molecules to the PSD that influence the clustering process. Furthermore, like neurotransmitter receptors, many components of the PSD are subject to multiple types of regulation by protein–protein and protein–lipid interactions, endowing synapses with dynamic properties that enable fast, enduring and reversible structural and functional adaptations to changes in network activity.

The aim of this Review is to discuss recent progress in understanding the formation and regulation of inhibitory synapses (that is, GABAergic and glycinergic synapses) in the vertebrate CNS. Our discussion focuses on gephyrin, which is the core scaffolding protein in inhibitory PSDs[1-3]. Gephyrin anchors, and thereby clusters, glycine receptors (GlyRs) and $GABA_A$ receptors ($GABA_ARs$) at postsynaptic sites[2] (BOX 1), interacts with multiple classes of signalling molecules (Supplementary Table 1, and undergoes complex post-transcriptional modification (BOX2). Here, we discuss these mechanisms and how they modulate gephyrin's clustering properties and functions to enable structural and functional regulation of inhibitory neurotransmission in response to a vast array of signalling events.

The main roles of inhibitory neurotransmission, especially GABAergic transmission, are to control neuronal excitability and to synchronize neuronal networks to generate oscillations that underlie cognitive processes. GABAergic transmission, acting mainly via $GABA_ARs$, probably affects every neuron in the CNS, whereas glycinergic transmission mainly operates in caudal CNS structures[4]. In addition, GABAergic transmission regulates major neurodevelopmental processes, and functional and structural alterations of GABAergic circuits contribute to the pathophysiology of various brain diseases, including autism-spectrum-related disorders, intellectual disabilities, schizophrenia, anxiety and major

depression[5,6]. Thus, we also discuss gephyrin's role as a master organizer of inhibitory PSDs in the context of CNS development, neuronal differentiation and major brain disorders.

Gephyrin and molybdenum cofactor synthesis

Gephyrin is a highly conserved, phylogenically ancient protein and shows widespread tissue expression in vertebrates[1,7]. Targeted deletion of *Gphn* (the gene encoding gephyrin) in mice revealed that gephyrin mediates molybdenum cofactor (Moco) biosynthesis[8], and this function has been ascribed to gephyrin homologues across species[9]. Moco chelates and activates molybdenum in the active centres of molybdenum enzymes, and one of the four known molybdenum enzymes in vertebrates — sulphite oxidase — is crucial for survival[10]. Mutations in *GPHN* can cause the rare genetic disorder Moco deficiency, which is fatal unless functional sulphite oxidase can be rescued[11].

Analysis of primary neuron and astrocyte cultures indicated that Moco synthesis in the CNS takes place only in astrocytes[12]. However, this *in vitro* finding, which implies that gephyrin's functions in neurons are related to the regulation of inhibitory neurotransmission, awaits confirmation.

Postsynaptic gephyrin clustering

Despite gephyrin's highly conserved role in Moco synthesis, this protein was discovered in purified preparations of mammalian glycine receptors (GlyR)[13], owing to its high binding affinity for the main intracellular loop of the GlyR β subunit. Indeed, gephyrin's name is derived from its ability to bind tubulin and, thereby, act as a bridge between GlyRs and the cytoskeleton[14].

Antibodies to GlyRs and gephyrin[15] allowed the first ultrastructural detection of a neurotransmitter receptor and its scaffolding protein in mammalian CNS tissue[16], as well as the discovery of mixed GABAergic–glycinergic synapses[17,18]. Subsequently, the presence of gephyrin in GABAergic-only synapses was reported in the retina[19] and later confirmed throughout the CNS[20,21]. In these studies, gephyrin immunostaining revealed the existence of brightly stained puncta (0.05–2 μm^2) that were selectively located at GABAergic and glycinergic postsynaptic sites (FIG. 1)[22]. As these puncta represented high local concentration of self-assembled gephyrin molecules, they were termed 'clusters'. In contrast to the GlyR β subunit, biochemical evidence for a direct interaction between gephyrin and $GABA_AR$

subunits proved difficult to obtain, suggesting that fundamental differences exist between gephyrin's roles as a scaffolding protein in glycinergic and GABAergic synapses.

The selective localization of gephyrin clusters at postsynaptic sites implies that neuron-specific molecular mechanisms ensure its targeting and postsynaptic clustering. The molecules that interact with gephyrin belong to various functional classes (Suppl. Table 1), and their specific roles and localizations in inhibitory synapses remain largely elusive (for recent reviews, see Refs [2,3,23-25]). Importantly, unlike the scaffolding molecules that form glutamatergic PSDs, gephyrin and the majority of its known interactors lack a canonical protein–protein interaction domain, such as the PDZ domain. Thus, it is unclear how the multi-molecular complexes that comprise the PSD of inhibitory synapses are formed and held together, and what prevents gephyrin to self-aggregate at non-synaptic sites (as it occurs when over-expressed in non-neuronal cells[26]). The identification of several motor proteins as gephyrin interactors also raised the possibility that a non-clustered form of gephyrin contributes to post-Golgi transport and cell surface delivery of GlyRs (reviewed in Ref [27]).

Elucidating how protein–protein interactions and post-translational modifications regulate gephyrin transport, scaffolding and clustering will be crucial for understanding the formation and maintenance of inhibitory synapses in the CNS. Below, we examine molecular composition differences between glycinergic and GABAergic synapses. We mainly discuss the role of gephyrin-interacting molecules in synapse formation and plasticity. This focus does not exclude the possibility that gephyrin exerts additional roles, for example, in axon terminals or the nucleus, but these potential roles will not be covered here.

Gephyrin and postsynaptic receptors

GlyRs and $GABA_A$Rs belong to the superfamily of Cys-loop ligand-gated ion channels and are made up of five subunits[28]. They share considerable structural homology but exhibit several major differences that are relevant for understanding the differential assembly and plasticity of inhibitory synapses.

Five GlyR subunit genes exist, encoding the α1–α4 and β subunits, and these subunits form either homomeric receptors (for the α subunits only) or α- and β-subunit-containing heteromeric assemblies. The stoichiometry of heteromeric GlyRs is still a matter of dispute, with studies reporting that such receptors comprise three α and two β subunits[29] or two α and three β subunits[30,31]. The distribution of GlyRs in neurons, as visualized by immunostaining

with antibodies against α subunits, perfectly overlaps with the distribution of gephyrin clusters, pointing to postsynaptic localization (FIG. 2A). The functional importance of the gephyrin–GlyR interaction, which relies on high-affinity binding between gephyrin and the third intracellular loop of the β subunit, is evident from the findings that silencing gephyrin expression precludes postsynaptic clustering of GlyR[32] and insertion of the binding motif of the GlyR β subunit into other transmembrane proteins is sufficient for those proteins to associate with gephyrin[33]. In line with these data, studies have found that gephyrin and GlyRs in transport vesicles can form intracellular associations[34,35].

In contrast to GlyRs, $GABA_A$Rs exhibit extensive subunit heterogeneity — 19 $GABA_A$R subunit genes exist, encoding the α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ and ρ1–ρ3 subunits — and display only low-affinity interactions with gephyrin. $GABA_A$Rs are typically assembled from at least three different classes of subunit (α/β/γ or α/β/δ). Morphological analysis has revealed that only a subset of $GABA_A$R subtypes, notably those containing the α1, α2 or α3 subunit along with the γ2 subunit, are localized postsynaptically with gephyrin[21] (FIG. 2B). Accordingly, the α1–α3 subunits interact with gephyrin[36-39] at the site that the GlyR β subunit binds to, but the former show with a 500-fold lower binding affinity[40]. By contrast, receptors containing the α4, α5 and δ subunit, which mainly form extrasynaptic $GABA_A$Rs, do not colocalize with gephyrin[41-45].

The role of gephyrin in the postsynaptic clustering of $GABA_A$Rs is not yet established. Importantly, targeted deletion of the γ2 subunit precludes the postsynaptic clustering of $GABA_A$Rs and gephyrin[46], although this subunit does not directly bind gephyrin. The mechanisms underlying this indirect effect might involve the reduced cell-surface expression of $GABA_A$Rs that contain α and β subunits only, and/or the existence of proteins that require the γ2 subunit to interact with gephyrin to enable its clustering. Furthermore, other studies involving the targeted deletion of *Gphn* or other subunit genes have found that gephyrin-independent $GABA_A$R clustering can occur and that the requirement for gephyrin for $GABA_A$R clustering is dependent on neuronal and synapse type[43,47-50].

Importantly, gephyrin itself depends on the presence of $GABA_A$Rs to form postsynaptic clusters in GABAergic synapses, again in a cell-specific manner. This feature was observed following targeted deletion of *Gabra1* (encoding the $GABA_A$R α1 subunit), which abrogates gephyrin clustering in cerebellar Purkinje cells and thalamic relay neurons[42], without influencing the distribution of extrasynaptic, α4-containing $GABA_A$Rs. In mutant neurons devoid of postsynaptic $GABA_A$Rs, gephyrin forms large aggregates that are located in the

soma or a dendrite; electrophysiologically, no synaptic GABAergic currents can be detected in these cells[51]. Nonetheless, some functionally silent GABAergic synapses can form[52,53]. Similar impairments in gephyrin postsynaptic clustering have been reported in *Gabra3* knockout mice[54]. A more complex situation was observed in *Gabra2* knockout mice, in which gephyrin clustering is largely abrogated in CA1 pyramidal cells, despite the retention of postsynaptic clusters of α1-containing $GABA_A$Rs. Thus, the postsynaptic clustering of gephyrin at GABAergic synapses depends on interactions between gephyrin and specific $GABA_A$R subtypes, indicating that these receptors have a fundamental influence on the molecular composition and function of the PSD. In this context, it is not fully clear why α4- and α5-containing $GABA_A$R do not interact with gephyrin; however, the α5 subunit interacts with radixin, a member of the ezrin/radixin/meosin protein family, enabling its anchoring to the actin cytoskeleton following radixin activation[55]. It is not established whether a similar mechanism also regulates the cell surface expression of other extrasynaptic $GABA_A$R subtypes (containing the α4 and/or δ subunit)[56].

The structural heterogeneity of $GABA_A$Rs confers variability in the molecular composition and functional properties of GABAergic synapses. This variability might be relevant for the functions of gephyrin. In addition, GABAergic synapses are associated with distinct signalling complexes (FIG. 2B, C) in specific neurons or subcellular compartments, suggesting additional functional heterogeneity. A prominent example is the dystrophin–glycoprotein complex (DGC)[57], which is associated selectively with a subset GABAergic synapses in cerebral cortex and cerebellum, where it regulates postsynaptic anchoring of $GABA_A$Rs[43], most likely independently of gephyrin. In striate muscle cells, where the DGC was characterized initially, it is essential for the functional integrity of the sarcolemma and the maintenance of the neuromuscular junction. However, besides mechanical stabilization of the sarcolemma during contraction, the DGC has a signalling role: specifically, it anchors signalling molecules, including syntrophins, dystrobrevins and neuronal nitric oxide synthase (nNOS), at specific locations in the plasma membrane (FIG. 2C). In neurons, mutations in the genes encoding dystrophin or dystroglycan can disrupt the DGC and impair postsynaptic clustering of $GABA_A$Rs, indicating that the DGC makes a direct contribution to the maintenance of inhibitory transmission. In addition, the DGC interacts with numerous molecules that influence gephyrin function and clustering properties and the formation of GABAergic synapses. These include synaptic adhesion molecules (such as neurexins, neuroligins (NLGNs) and the synaptic scaffolding molecule (S-SCAM)[58]) and signalling molecules, including nNOS and SynArfGEF[59] (FIG. 2C). Although these interactions have

not been investigated in detail to date, the preliminary evidence suggests that DGC has a key role in the regulation of gephyrin scaffolds and GABAergic synapse function (reviewed in Ref. [60]).

Key regulators of gephyrin clustering

Neuroligin 2. NLGNs are adhesion molecules that have fundamental roles in the regulation of synapse formation and function. Along with their trans-synaptic partners, the neurexins, they might trigger, or at least facilitate, the formation of synapses, as shown most impressively by co-cultures of neurons and non-neuronal cells overexpressing NLGNs or neurexins, in which synapse formation is enhanced by these molecules[61-63]. Among the four NLGNs, which are encoded by distinct genes, NLGN2 and to a lesser extent NLGN4 are selectively associated with GABAergic and glycinergic synapses, respectively[64,65]. NLGN3, which interacts with both NLGN1 and NLGN2[66], has been reported to be present in some GABAergic and possibly glycinergic synapses[67,68] (FIG. 2A-B), but its role in these structures is not established. Among synaptic adhesion molecules implicated in GABAergic synapse formation, NLGN2 is the only one known to interact with gephyrin (but see Ref. [69]) at GABAergic synapses. Thus, it is thought that NLGN2 drives the formation of gephyrin and $GABA_AR$ postsynaptic clusters at nascent GABAergic postsynaptic sites[70]. Targeted deletion of the genes encoding NLGNs has not been able to fully reveal their function[71], presumably because of the existence of compensatory mechanisms involving functionally homologous synaptic adhesion molecules. However, in triple-mutant mice both GABAergic and glycinergic synapses were severely altered in brainstem respiratory networks. Moreover, *Nlgn2*-null mice exhibit region-specific alterations in GABAergic synapses. In these mice, there is a loss of $GABA_AR$ (but not GlyR and gephyrin) clusters in the retina, causing impaired light processing[72], whereas in the hippocampus and dentate gyrus, gephyrin and $GABA_AR$ clusters are selectively lost in perisomatic but not dendritic synapses of principal cells[70,73]. In the cerebral cortex, absence of NLGN2 results in decreased inhibitory transmission from fast-spiking parvalbumin interneurons (which form perisomatic and axo-axonic synapses) but not from somatostatin interneurons (which target distal dendrites)[74]. *Nlgn2*-knockout mice also exhibit increased anxiety-like behaviour and high reactivity to handling, compatible with a decrease in inhibitory transmission in brain regions that regulate emotional behavior[75,76].

Collybistin. Collybistin (CB), encoded by *Arhgef9*, was found to interact with gephyrin in a yeast two-hybrid screen and to translocate gephyrin towards the cell surface in non-neuronal cells[77]. CB is a neuron-specific guanine nucleotide exchange factor (GEF) of the DBL family. Members of this family are characterized by three functional domains — a N-terminal type 3 src homology domain (SH3), a catalytic DH domain and a PH domain — and activate small GTPases of the RHO family[78]. Indeed, CB selectively activates the RHO family member CDC42[79].

CB directly binds gephyrin's C-terminal domain[80], and its binding site overlaps with those of the $GABA_AR$ α2 and α3 subunits[37]. Gephyrin binds to CB's DH domain[81] and may potentially interfere with CB's ability to activate CDC42[79].

CB pre-mRNA is subject to alternative splicing, giving rise in rats to three isoforms that differ only in the C-terminus; further mRNA splicing determines the presence or absence of the N-terminal SH3 domain in each of these isoforms (reviewed in Ref. [60]). The relevance of this heterogeneity is not clear. The expression of multiple CB splice isoforms in neurons suggests that they may have non-overlapping functions, albeit with some redundancy; however, it is not known whether CB isoforms differ in their spatiotemporal expression patterns in the CNS (or in individual neurons) and which factors determine alternative splicing of CB pro-mRNA. It is also unknown whether CB splicing sites in rats are fully conserved across species; the only CB isoforms known to date in human, encoded by hPEM2, correspond to $CB3_{SH3}^{+}$ and $CB3_{SH3}^{-}$ in rats[82].

Of the two isoforms that were initially described to interact with gephyrin, $CB1_{SH3}^{+}$ and $CB2_{SH3}^{-}$, only the latter was able to translocate gephyrin to the cell surface[77], suggesting that the SH3 domain controls CB activity by auto-inhibition of its catalytic domain. However, this dichotomy is not apparent following overexpression of specific CB isoforms in neurons, and there is a consensus that all the main CB isoforms are capable of gephyrin clustering when they are overexpressed in cultured neurons[83-85]. Accordingly, CB has been detected at GABAergic postsynaptic sites in rodent brain[86] (FIG. 2B).

A fundamental insight into CB's role in gephyrin clustering was provided by targeted deletion of *Arhgef9*[87], which revealed that CB is dispensable for gephyrin (and GlyR) clustering in glycinergic synapses. Accordingly, *Arhgef9*-null mice do not exhibit glycinergic synaptic dysfunction, and are viable and fertile. It is not known, however, whether the absence of CB is compensated for by a functionally related mechanism. In the GABAergic system, cell- and synapse-specific alterations were observed in *Arhgef9*-null mice. Although no effects were apparent in hippocampal interneurons or cerebellar granule cells, clustering of

gephyrin, but not $GABA_A$Rs, was disrupted in Purkinje cells, and clustering of both gephyrin and $GABA_A$R was impaired in hippocampal and cortical pyramidal cells. Functionally, GABAergic transmission was reduced in the hippocampal formation in *Arhgef9*-null mice, leading to enhanced long-term potentiation (LTP). Behaviourally, these mice showed increased signs of anxiety and impaired spatial learning, compatible with a reduction in inhibitory neurotransmission. Interestingly, the same functional alterations could be induced by conditional inactivation of *Arhgef9* after the period of synaptogenesis, implying that CB is also required for GABAergic synapse maintenance[88].

Role of cytoskeletal and motor proteins

Strong evidence indicates that GlyRs are transported intracellularly to and from glycinergic synapses by forming a complex with gephyrin that is bound to the kinesin family motor protein 5 (KIF5) or to dynein-light chain 1 (DLC1) or DLC2. These complexes suggest that gephyrin serves as a motor-cargo adaptor protein (reviewed in Ref. [27]). Its role in $GABA_A$R trafficking to synapses is less clear. Such trafficking might involve formation of a complex of KIF5 with $GABA_A$R-associated protein (GABARAP) and gephyrin[89] (but see Ref.[90]). Alternatively (or in addition), $GABA_A$R trafficking is mediated by KIF5 in complex with Huntingtin-associated protein 1 (HAP1)[91]. Because gephyrin splice variants that contain the G2 cassette preclude clustering of GlyRs at GABAergic synapses[92], it is conceivable that gephyrin's structure determines its binding to specific motor protein complexes to ensure the proper sorting of receptors to appropriate synapses.

Nevertheless, only limited knowledge exists about how gephyrin interacts with the cytoskeleton (microtubules and microfilaments) for intracellular transport and local clustering. Indeed, the significance of gephyrin binding to tubulin remains unclear, given that postsynaptic densities mainly contain actin microfilaments and only a few microtubules. Disruption of microtubules in cultured spinal cord neurons (which receive a mixture of glycinergic and GABAergic inputs) caused a rapid reduction of gephyrin clusters[93,94], but this effect depends on neuronal maturity[95]. Moreover, disruption of the tubulin or actin cytoskeleton in mature cultured hippocampal neurons did not affect gephyrin clustering[96]. More recently, gephyrin-mediated cell surface delivery and postsynaptic clustering of GlyRs was shown to be regulated by GlyR- and activity-dependent post-transcriptional modifications of tubulin[97]. This result, again obtained in cultured hippocampal neurons, stands in stark contrast with a report that chronic blockade of glycinergic transmission onto motor neurons does not affect gephyrin clustering *in vivo*[98]. Methodological differences (especially in

preparing and maintaining primary neuronal cultures), as well as differential regulation of GABAergic versus glycinergic synapses (in dually innervated cells), might contribute to these controversial results. Moreover, the view that gephyrin acts as a motor–cargo adaptor protein is probably incomplete, since gephyrin trafficking to GABAergic postsynaptic clusters crucially depends on collybistin function.

With regard to the actin cytoskeleton, gephyrin has been shown to interact with membrane-anchored profilin 1 and profilin 2 (two neuronally enriched isoforms of this monomeric actin-binding protein), and these complexes can interact with microfilament adaptors of the mammalian enabled (Mena)/vasodilator stimulated phosphoprotein (VASP) family[99,100]. In cultured cortical neurons, profilin 2A (an isoform of profilin 2) shows extensive co-localization with postsynaptic gephyrin clusters, and its abundance at postsynaptic sites is modulated by neurotrophins and neuronal activity[101]. The gephyrin–profilin interaction has been proposed to regulate dynamic modifications of the postsynaptic actin cytoskeleton, either up- or down-stream of gephyrin anchoring to the PSD. Alternatively, profilins may interact with components of the endocytotic machinery, thereby potentially contributing to trafficking and turnover of synaptic proteins[102]. In this context, removal of $GABA_A$Rs from the synapse by endocytosis probably occurs independently of gephyrin, and requires interactions between $GABA_A$Rs and the clathrin-adaptor protein AP2[103] and, subsequently, muskelin, which also associates with the dynein motor complex[104].

Finally, the functional importance of the interactions between gephyrin and DLCs should remain open for reinterpretation for the following reasons. First, it is unclear whether the gephyrin–DLC1 association to the dynein motor complex might serve to transport gephyrin between postsynaptic clusters or to traffic endocytosed GlyRs[105]. Second, expression of a mutant variant of gephyrin that lacks the DLC interaction site in its C-domain does not affect gephyrin postsynaptic clustering in primary neurons[106], calling into question the role of this interaction in gephyrin transport. Third, DLC1 is also known as protein inhibitor of nNOS (PIN) and interacts with nNOS to inhibit its activity[107]. Since nNOS has been detected in specific GABAergic synapses[108,109] (FIG. 2C), it is conceivable that the gephyrin–DLC1 interaction serves to regulate nNOS activity rather than enabling retrograde transport of gephyrin.

Mechanisms of gephyrin clustering

Molecular organization of glycinergic and GABAergic PSDs. A quantitative analysis of gephyrin molecules in the PSD involving single-molecule imaging methods[110] revealed that

gephyrin clusters are located at postsynaptic sites in spinal cord neurons and comprise about 3,000–10,000 gephyrin molecules. In addition, this study revealed the presence of non-synaptic micro-aggregates, suggesting that postsynaptic clusters are formed by local accumulation of these micro-aggregates; the packing density of gephyrin molecules was higher in synapses containing high amounts of GlyRs than in those enriched with $GABA_A$Rs, suggesting that glycinergic and GABAergic synapses have distinct differences in gephyrin scaffold organization and molecular composition.

In line with this assumption, analysis of activity-dependent changes in $GABA_A$R and GlyR clustering in mixed synapses revealed that chronic tetrodotoxin administration had no effects on gephyrin and GlyR clustering but did reduce the number of $GABA_A$Rs. This finding was also in line with the fact that direct molecular interactions of $GABA_A$Rs and GlyRs with the gephyrin scaffold influence their spatial and temporal synaptic confinement[111], albeit through distinct mechanisms that are regulated by activity-dependent processes[112,113]. In particular, for both types of receptors, phosphorylation of specific subunits influences the binding affinity of the receptor complexes to gephyrin and thereby their postsynaptic clustering properties[38,114]. Importantly, evidence indicates that gephyrin clustering can be perturbed without concomitant effects on GlyR postsynaptic aggregation[115], pointing to the existence of independent regulation mechanisms for postsynaptic receptor clustering.

The receptor activation model. The first model that attempted to explain the role of gephyrin for GlyR (and $GABA_A$R) clustering at postsynaptic sites postulated that presynaptic neurotransmitter release induced membrane depolarization due to chloride efflux, which led to Ca^{2+}-mediated aggregation of gephyrin and, in turn, binding of gephyrin to the cytoskeleton and immobilization of GlyRs in the PSD[116]. In addition, binding of gephyrin to membrane-anchored CB was proposed to regulate actin dynamics (via CDC42 activation) and downstream signalling. This model was further refined to include activation of phosphatidylinositol-3 kinase (PI3K) by extracellular signals to promote the formation of PtdIns(3,4,5)P_3 and thereby membrane anchoring of CB and profilin[117]. Metabotropic receptors and tyrosine kinase receptors, such as the insulin receptor, were proposed to activate PI3K. However, a major question unanswered by this model was that chronic antagonism of $GABA_A$Rs (that is, in the absence of Cl^--mediated neuronal depolarization, postulated in the first step of the model) does not prevent synaptogenesis *in vitro*, indicating that alternative mechanisms must exist to enable synapse formation.

Subsequently, a key role in gephyrin clustering at inhibitory synapses was attributed to CB, rather than receptor activation. It was demonstrated that the interaction between CB and gephyrin and the presence of the PH domain of CB are both required for gephyrin clustering at GABAergic synapses[80,82]. Moreover, this study showed that the G55A CB mutation, identified in a patient with severe hyperekplexia, impairs gephyrin clustering, providing a mechanistic explanation for the symptoms of this patient. A more recent study provided evidence that this point mutation affects CB conformation, disrupting the PH domain function and thereby gephyrin clustering[85].

Gephyrin clustering at glycinergic synapses. The observation that *Arhgef9*-null mice do not exhibit postsynaptic defects at glycinergic synapses indicated that multiple mechanisms regulate gephyrin clustering in various types of inhibitory synapses. To date, no model has replaced the 'receptor activation model' discussed above, and it is not known which cell-adhesion molecules, in particular among the NLGNs[71], are required for gephyrin clustering at glycinergic synapses (FIG. 2A). It is generally assumed that GlyR clusters bound to gephyrin form upon intracellular trafficking of GlyRs bound to gephyrin, followed by regulated lateral diffusion of GlyRs in the plasma membrane[27]. In addition, several mechanisms have been identified that regulate gephyrin binding to GlyRs. For example, PKC-dependent phosphorylation of Ser403 in the β subunit reduces its binding affinity to gephyrin, causing dispersion of the receptors in the plasma membrane[114]. Furthermore, postsynaptic accumulation of GlyRs in spinal cord neurons and their anchoring to the gephyrin scaffold is regulated by integrins β1 and β3, by a mechanism involving Ca^{2+}-calmodulin kinase 2 (CaMKII) activity[115]. However, the substrate targeted by CaMKII has not been identified. Finally, manipulating the levels of heat shock cognate protein 70 in cultured neurons affects gephyrin clustering at postsynaptic sites (FIG. 2A), without influencing GlyR accumulation, implying that both processes might be uncoupled[118]. It should be noted, however, that these various data provide no mechanistic explanation for the formation of glycinergic synapses, and their interpretation is limited by the fact that the spinal cord neurons used for analysis contain a large fraction of mixed glycinergic–GABAergic synapses among their inhibitory synapses. Thus, it is unclear whether the findings reported so far would only hold true for pure glycinergic synapses or mixed glycinergic–GABAergic synapses.

Gephyrin clustering at GABAergic synapses. A compelling model to explain gephyrin clustering during GABAergic synapse formation was put forward based on the ability of NLGN2 to interact with both the SH3 domain of CB and gephyrin. According to this view,

NLGN2 binding to CB releases the SH3 domain-mediated CB auto-inhibition, thereby triggering further recruitment and eventually clustering of gephyrin and $GABA_A$Rs in the nascent PSD[24,70]. An alternative theory, however, states that a trimeric complex first forms between gephyrin, CB and α2-containing $GABA_A$Rs, and that the latter 'activates' the CB SH3 domain[119] to enable formation and concerted growth of the postsynaptic gephyrin and $GABA_A$R clusters. Both models offer an explanation for the loss of gephyrin and NLGN2 clustering in neurons lacking the $GABA_A$R α2 subunit[60], and they emphasize the need for the presence of (or signalling by) specific $GABA_A$Rs for proper formation and maintenance of a GABAergic PSD.

However, these two models fail to explain the heterogeneity of the gephyrin clustering phenotype of *Arhgef9*-null mice, such as the preservation of gephyrin clusters, or $GABA_A$R clusters, in distinct subsets of neurons[87]. They also do not take into account a possible role of activated CDC42 for enabling or modulating gephyrin clustering[85]. Their central assumption that CB activation is required for gephyrin recruitment to GABAergic synapse needs confirmation, as the nature of this activation is still unclear. Intriguingly, gephyrin clustering is not impaired in the *Cdc42*-null mice or in neurons expressing a catalytically inactive CB mutant[120]. Moreover, co-transfection studies in cultured hippocampal neurons have demonstrated that 'inactive' $CB2_{SH3}^{+}$ is at least as efficient as 'active' $CB2_{SH3}^{-}$ at promoting the formation of postsynaptic gephyrin clusters[83,84]; and co-expression of constitutively active or dominant-negative CDC42 with CB2 isoforms dramatically affects the shape and size of postsynaptic gephyrin clusters[85]. By contrast, the PH domain of CB, which anchors it to phosphatidylinositol (3,4,5)-triphosphate ($PtdIns(3,4,5)P_3$) in the plasma membrane (FIG. 2B), is required for gephyrin clustering. In its absence, gephyrin is trapped in long filamentous structures in the soma and dendrites of transfected hippocampal neurons[85]. Of note, overexpression of constitutively active CDC42 can rescue this phenotype, indicating that CDC42 either facilitates CB function or can compensate for it.

Taken together, it appears that CB isoforms, independently of the SH3 domain but in cooperation with CDC42, stabilize intracellular gephyrin and contribute to its transport to postsynaptic sites. Extending this concept, it is conceivable that gephyrin binding to CB is necessary to prevent its self-aggregation outside of inhibitory PSDs; it might thus facilitate its intracellular transport and cell-surface targeting, and may serve to control CDC42 activation[79]. Hence, CB isoforms probably regulate multiple facets of gephyrin clustering, both outside and inside the GABAergic PSD; furthermore, the concept that the SH3 domain serves to regulate CB enzymatic activity needs to be revisited.

To fully comprehend the mechanisms regulating gephyrin clustering at GABAergic synapses, it will ultimately be necessary to take into account the molecular diversity of such synapses, both between neurons and within specific subcellular sites (FIG. 2B, C). Molecular diversity at GABAergic synapses ensues primarily from the heterogeneity in the subunit composition of $GABA_ARs$. It also arises from the subcellular localization of GABAergic synapses on soma, dendrites, axon initial segment and spines, as each of these compartments provides a specific molecular microenvironment in which the synapses are embedded. Subcellular localization is also associated with diversity of inputs; this is best demonstrated in the hippocampus, where over 20 distinct interneuron subtypes, each with a specific innervation pattern, have been identified[121]. Molecular diversity is also exhibited at inhibitory PSDs through heterogeneity in their constituent proteins (reviewed in Ref. [60]) and by the diversity of posttranslational modifications, which probably regulate many, if not all, proteins at GABAergic PSDs (BOX 2). Finally, as presented in BOX 3, multiple transcriptional and translational control mechanisms might have crucial roles by determining the availability of proteins required for regulating gephyrin clustering and scaffolding properties.

Gephyrin and GABAergic synapse formation

The ability of gephyrin to interact with specific transsynaptic adhesion molecules (Suppl. Table 1) poses the question about its roles during the initial steps of synapse formation. In particular, is gephyrin involved in determining the neurotransmitter specificity of inhibitory synapses by ensuring the accumulation of the 'correct' neurotransmitter receptors in the PSD? The apparent segregation of NLGN isoforms between glutamatergic, GABAergic and glycinergic synapses suggests that neurexin–NLGN interactions have a major role in this segregation. Therefore, understanding how, when, and where gephyrin interacts with NLGN isoforms will be important to understand the specification of glycinergic and GABAergic synapses. Unexpectedly, recent evidence indicates that the differential association of NLGN isoforms with PSD-95 or gephyrin is a regulated process that depends on the tyrosine phosphorylation of identified NLGN residues[69]. Furthermore, NLGN dimerization seems to be essential for its cell-surface function[66] and for instructing the differentiation of the presynaptic terminal[122], providing another mechanism for regulating its interactions with synaptic scaffolding molecules. Recently, the MAM domain-containing GPI anchor proteins MDGA1 and MDGA2 (which are Ig superfamily adhesion molecules) were shown to selectively bind to NLGN2 and interfere with its synaptogenic activity[123,124] (FIG. 2D). It will

be interesting to determine whether these proteins interact with gephyrin while mediating these effects.

To understand the contribution of gephyrin to the formation of GABAergic synapses, one also has to take into account that modulating the phosphorylation status of gephyrin is sufficient to regulate within hours the density of GABAergic synapses on dendrites in cultured neurons and *in vivo* (BOX 2)[125]. This key observation indicates that signalling cascades that control gephyrin phosphorylation regulate not only its postsynaptic aggregation but also its interactions with transsynaptic cell adhesion molecules with synaptogenic functions. We propose that GABAergic synapse formation might require membrane anchoring of CB and the activation of PI3K–Akt signalling, leading to the downstream inhibition of GSK3β and a reduction in gephyrin phosphorylation at Ser270 (FIG. 2D)[125]. De-phosphorylation of this residue is associated with a conformational change in gephyrin[126], which might be crucial for interacting with and activating synaptogenic molecules.

Interactions between gephyrin and $GABA_A$Rs also contribute to the integrative function of gephyrin in controlling GABAergic synapse formation. This mechanism has been demonstrated elegantly in a recent study that involved mosaic deletion of *Gabrg2* in the CNS[127]. This experiment resulted in reduced postsynaptic clustering of $GABA_A$Rs (and other components of the PSD) in *Gabrg2*-null neurons and a compensatory increase in GABAergic synapses in neighbouring non-mutated cells. Thus, neurons compete with each other for presynaptic terminals to engage in synaptic inhibition, and this process depends, in turn, on their ability to form functional gephyrin-containing PSDs. A role for the BDNF receptor, TrkB, in this process, is conceivable, as it has been shown to control GABAergic synaptogenesis downstream of NLGN2 by influencing gephyrin clustering[128].

Finally, alternative pathways have been identified to selectively mediate the formation of GABAergic synapses[129], opening the possibility that complementary mechanisms cooperate to ensure proper development and differentiation of the PSD. Such diversity might be important for governing GABAergic synapse formation in diverse subcellular compartments, notably in dendritic spines[130,131] and in the axon-initial segment[43], which have a specialized cytoskeleton. Molecular diversity is even present within single synapses, with $GABA_A$Rs and gephyrin being restricted within the GABAergic PSD to microdomains complementary to those occupied by the adhesion protein IgSF9b, which is coupled to NLGN2 by means of binding to S-SCAM[132] (FIG. 2D).

Gephyrin and GABAergic synaptic plasticity

As a global increase in network activity is sufficient to activate ERK and decrease the size of gephyrin clusters (by a mechanism involving calpain)[133] (see BOX2), the phosphorylation status of gephyrin may act as a sensor to adjust the strength of inhibitory transmission in response to changes in network activity. Furthermore, the ability of gephyrin to anchor signalling molecules — notably CB, SynArfGEF and their effectors — and actin-modifying molecules at inhibitory PSDs implies that it regulates downstream signalling, with major potential consequences for synapse function and plasticity (FIG. 3).

In addition to gephyrin posttranslational modifications, modifications of $GABA_AR$ subunits also have an essential role in regulating GABAergic synapse function and plasticity. For example, γ2 subunit palmitoylation is important for $GABA_AR$ postsynaptic clustering[134], whereas ubiquitination of a motif within the γ2 intracellular domain regulates the targeting of such receptors to the degradation pathway.[135] Serine/threonine and tyrosine phosphorylation also modulate trafficking and cell surface expression (as well as the gating properties) of specific $GABA_AR$ subtypes (reviewed in Ref. [136]), thereby contributing potentially to synaptic plasticity mechanisms. In future studies, it will be crucial to determine whether phosphorylation of $GABA_ARs$ is functionally coupled to gephyrin phosphorylation, either by the same or by another effector. Given the high number of potential phospho-residues in gephyrin (BOX 3), both possibilities are likely.

Gephyrin might act as a signalling hub, integrating excitatory synaptic activity and a variety of extracellular signals to adjust the strength of GABAergic (and possibly glycinergic) transmission by adapting the structure and functional properties of the scaffold anchoring receptors (FIG. 3), and thereby maintaining inhibitory–excitatory balance. Accordingly, extracellular signals can act on gephyrin scaffolds by mobilizing protein kinase cascades and intracellular Ca^{2+} stores to modify the function (and potentially number) of GABAergic synapses over a short time scale. Changes in the gephyrin scaffold might also impact its interactions with signalling molecules, and therefore produce structural and functional adaptations down-stream of the GABAergic PSD (FIG. 3). There is evidence for rapid changes in size and location of postsynaptic gephyrin clusters in cortical projection neurons in response to alterations in excitatory activity *in vitro*[137,138] and also in response to sensory deprivation during the critical period of visual cortex maturation[131,139]. A recent study confirmed that manipulation of GSK3β activity, targeting gephyrin residue Ser270, regulates dendritic growth and branching by altering GABAergic, but not glutamatergic transmission[140].

The abundant literature demonstrating changes in GABAergic transmission upon activation of metabotropic or tyrosine kinase receptors, as well as LTP/LTD at GABAergic synapses[141,142], has not yet implicated gephyrin as potential molecular substrate that contributes to the regulation of postsynaptic $GABA_AR$ availability[143]. However, studies showing translocation of effectors, such as CaMKII or calcineurin, from glutamatergic to GABAergic synapses[144,145] suggest that such effectors might interact not only with $GABA_ARs$ but also with gephyrin. Novel tools allowing endogenous PSD proteins in neurons to be tagged with fluorescent molecules without perturbing their cellular localization and function will enable direct visualization and quantification of changes in gephyrin clustering during alterations in inhibitory–excitatory balance[146].

Through the use of adult neurogenesis as a model system, we have shown that targeted deletion of the $GABA_AR$ $\alpha2$ subunit in adult-born olfactory bulb granule cell precursors leads to delayed loss of inhibitory synaptic currents, disruption of gephyrin clusters in the PSD, and major morphological impairments affecting the formation of glutamatergic synapses[147]. Although this study showed the importance of proper GABAergic synaptic transmission for neuronal development, it also suggested that a possible contributing mechanism to the morphological impairments might be related to the loss of gephyrin clusters, which might also affect their associated signalling molecules, notably those interacting with the cytoskeleton. According to this view, the gephyrin scaffold in the PSD would assume a role not only in anchoring receptors and related effector molecules but also in enabling local downstream signalling for neuronal development and differentiation, thereby giving a new dimension to the concept of GABAergic synapse plasticity and to the relevance of gephyrin as a synaptic scaffolding molecule.

Gephyrin and diseases of the nervous system

Only isolated reports exist of neurological diseases linked to *GPHN* mutations (for example, Moco deficiency[148], Stiff-Person syndrome[149], and Startle disease (hyperekplexia)[150]). The rarity of such diseases is presumably explained by the central role of glycinergic transmission, and hence gephyrin's postsynaptic function, in breathing, suckling and swallowing. In support of this hypothesis, restoration of Moco synthesis is not sufficient to rescue lethality in *Gphn*-null neonatal mouse pups[151]. Moreover, these diseases can also be caused by mutations affecting other proteins in the inhibitory PSD and that alter, but do not

abolish, glycinergic transmission; for example, hyperekplexia may be caused by mutations in genes encoding GlyR subunits, glycine transporters or CB[152].

GPHN mRNA is subject to extensive alternative splicing, implying that alterations of the *GPHN* splicing machinery may have a role in disease states. Indeed, aberrant alternative splicing has been observed in patients with temporal lobe epilepsy and is linked to expression of a gephyrin variant with a truncated G-domain, which alters gephyrin–$GABA_AR$ clustering[153]. Importantly, this study showed that an increase in cellular stress could induce exon skipping in *GPHN* and thus could provide a possible therapeutic target to prevent these alterations.

Experimentally, mutations in $GABA_AR$ subunit genes that affect gephyrin postsynaptic clustering have been used to generate models of neurodevelopmental and psychiatric disorders, including anxiety, major depression and schizophrenia[154,155]. For example, heterozygous conditional deletion of *Gabrg2* in mice causes diverse behavioural alterations that result in vulnerability of the brain to changes in GABAergic transmission during specific developmental time windows[156,157]. The significance of these results in the present context is that the perturbation of GABAergic transmission caused by *Gabrg2* deletion does not necessarily induce acute synaptic dysfunction. Rather, it induces alterations in the neuronal networks that are established during these critical periods[156], possibly due to impaired gephyrin clustering and downstream signalling. Taken from a broader perspective, a major implication of the complex regulation of gephyrin clustering is that mutations and disease mechanisms interfering with the proteins and signalling cascades that regulate gephyrin clustering have the potential to perturb GABAergic transmission during critical periods of brain development[158], and thereby to contribute to the pathophysiology of neurodevelopmental disorders[5,6,159,160]. Transcription factors, such as NPAS4, have been identified that regulate GABAergic synaptogenesis by controlling the expression of activity-dependent genes, and this finding may represent a potential mechanism for such perturbations[161] (BOX 3).

The modulation of gephyrin clustering properties, and thereby the strength of inhibitory transmission, by specific signalling cascades also has implications for therapeutic approaches that target these molecular pathways to restore inhibitory–excitatory balance or adjust GABAergic transmission in specific circuits. We have shown that chronic treatment with lithium, used in bipolar disorder therapy, effectively increases the density and size of gephyrin clusters in cultured neurons and in mice *in vivo*[125]. Although it is not yet established whether this effect contributes to lithium's therapeutic efficacy in patients with bipolar disorder, these

findings provide a proof-of-principle for the region- and drug-specific modulation of GABAergic transmission by the targeting of gephyrin phosphorylation.

Conclusions

This Review highlights how the view derived from early biochemical analysis of gephyrin, according to which gephyrin serves as a passive anchor holding GlyRs in the PSD of glycinergic synapses, has evolved with the identification of interacting molecules and post-translational modification mechanisms. The current evidence assigns a major role to gephyrin — in addition to its role in Moco synthesis — in the formation a protein scaffold that regulates inhibitory neurotransmission in a dynamic fashion, over a time scale of minutes to hours, in response to a large variety of extra- and intracellular signals. Specifically, we propose that gephyrin contributes to synaptic homeostatic plasticity by adapting the strength of GABAergic transmission to local changes in excitatory transmission. Furthermore, gephyrin might regulate neuronal differentiation and morphological plasticity by anchoring signalling molecules that interact with the cytoskeleton and membrane-bound effectors at specific subcellular sites[140]. These functions of gephyrin can take place at the PSD, as well as in other subcellular localizations, including dendritic spines, in particular when considering that gephyrin interacts with both anterograde and retrograde transport machineries.

This Review also underscores how fragmentary our understanding is of the mechanisms regulating gephyrin postsynaptic clustering, notably with regard to molecular heterogeneity of inhibitory synapses. At present, there is no satisfactory model to explain how gephyrin molecules assemble into a postsynaptic cluster; and the roles of gephyrin for cell-surface delivery and regulation of membrane diffusion of $GABA_A$Rs and GlyRs remain largely enigmatic and controversial. A better understanding of these complex mechanisms will require the identification of all molecules with synaptogenic potential at GABAergic and glycinergic synapses; clarification of the interactions between key players and their roles for mediating assembly of the gephyrin scaffold, synapse formation and plasticity, at both GABAergic and glycinergic synapses; and elucidation of the transcriptional and posttranslational regulation of these molecules.

The relevance of this quest lies in the contribution of GABAergic transmission to the pathophysiology of major neurological, neurodevelopmental and psychiatric disorders. We surmise that defective GABAergic signalling due to abnormal gephyrin clustering or function,

in particular during critical periods of brain development, might be a key factor for the development of these pathologies.

BOX 1: Gephyrin structure

In vertebrates, gephyrin comprises three structural domains (G, C and E domains). The G and E domains correspond to MogA and MoeA proteins that are responsible for Moco synthesis in bacteria. The C domain links the G and E domains and harbours numerous sites for post-translational modification and interaction with proteins regulating synapse formation and function[2]. Gephyrin more efficiently synthesizes Moco than do the isolated G and E domains[162], indicating an evolutionary advantage for protein fusion and structural rearrangements that gave rise to vertebrate gephyrin and allowed novel functions to emerge from the fused protein. In particular, gephyrin possesses a non-conserved surface-exposed loop in the E domain, which regulates its postsynaptic clustering in an all-or-none fashion[22]. This loop harbours a consensus sequence for protein phosphatase 1 binding, but the relevance of such binding remains unclear. Gephyrin is subject to extensive alternative gene splicing[12,150,163-166] – with well conserved gene structure between human and rodents[167] – and protein post-translational modifications (BOX 2), indicative of cell- and tissue-specific mechanisms regulating its functions for Moco synthesis and as a postsynaptic scaffolding molecule. In hepatocytes, for instance, predominant gephyrin splice variants contain the C3 cassette and are enriched in the cytosolic fraction, being part of a multi-protein complex[7].

Analysis of gephyrin structure is hampered by the instability of full-length gephyrin in solution. Biochemical and structural analysis revealed self-aggregation of the isolated G and E domains (and their homologues in bacteria and plants), resulting in formation of G domain trimers and E domain dimers[168,169]. Furthermore, the binding site of the GlyR β loop has been mapped to the E domain[170,171]. Accordingly, a model of hexagonal gephyrin lattice forming a two-dimensional scaffold anchoring GlyR in the plasma membrane was proposed[165,172], although this has now been called into question[2]. Analysis of recombinant gephyrin expressed in insect (Sf9) cells favoured the existence of hexameric gephyrin macro-molecular complexes and confirmed splice-variant specific gephyrin aggregation properties[173]. However, a recent structural analysis of gephyrin (expressed in *E. coli*) using atomic force microscopy and synchrotron X-ray scattering revealed that gephyrin forms trimers with multiple conformations, owing to partial flexibility of the C domain, and confirmed that the linker domain prevents E domain dimerization[174], as was shown previously[164]. Visualization of gephyrin clusters in glycinergic synapses using super-resolution microscopy revealed a 1:1 stoichiometry with GlyRs[110]. These observations place clear constraints on models of gephyrin scaffold organization, notably given the overwhelming evidence that GlyR β subunit loop binding occurs at the interface between two E domains. For example, the proposed hexameric gephyrin complex, but also the hexagonal gephyrin lattice model derived from structural studies[172] would not be compatible with a 1:1 gephyrin to β subunit stoichiometry, but only with a 2:1 stoichiometry. Furthermore, a 1:1 stoichiometry, with each gephyrin molecule having a similar probability to interact with a GlyR, implies a two-dimensional organization of the gephyrin scaffold that is aligned below the postsynaptic membrane containing the receptors.

BOX 2 Post-translational modifications of gephyrin

Post-translational modification of gephyrin may be important for determining its functions and localization at inhibitory PSDs. Such modifications might affect the structure and scaffolding properties of gephyrin, its trafficking and half-life, and indeed its ability to interact with partner proteins.

It has become increasingly clear that there are multiple phosphorylation residues on gephyrin[133,173,175,176,168,169], schematically mapped in Supplementary Figure 1 based on comparative results from four mass-spectroscopy analyses. This feature points to a role for specific interconnected kinase networks in regulating gephyrin function independently of its expression levels[177].

Among these residues, we have demonstrated that gephyrin residues Ser270 and Ser268 are two phosphorylation sites targeted by GSK3β and ERK1/2, respectively, that can synergistically influence the amplitude and frequency of GABAergic mIPSCs via gephyrin clustering changes. Thus, signalling pathways regulating gephyrin clustering properties can alter the strength of GABAergic transmission. Furthermore, the observation of collybistin-dependent phosphorylation of gephyrin Ser270 by cyclin dependent kinases, like CDK5[126], suggested convergence of signalling pathways on this critical residue. In line with these findings, a comprehensive screen of kinases for effects on gephyrin clustering in a recombinant expression system identified several candidates that are involved in signalling pathways activated by neurotrophins and growth factors[178]; these authors confirmed that alterations of gephyrin clustering were reflected in corresponding changes in GABAergic inhibitory transmission.

Much less evidence is available on the phosphatase(s) controlling gephyrin dephosphorylation. A direct interaction between gephyrin and protein phosphatase 1 has been observed in co-immunoprecipitation experiments, whereas application of broad spectrum phosphatase inhibitors to cultured neurons reduced postsynaptic gephyrin clustering[179], an observation opposite to our studies, in which gephyrin dephosphorylation increased postsynaptic clustering[125,133]. The serine/threonine residues potentially targeted by protein phosphatase(s) remain unknown.

In addition to phosphorylation, palmitoylation and acetylation of gephyrin have also been reported[133,180]. Palmitoylation, in addition to anchoring a protein to the membrane, can be essential for its functional properties. Thus, gephyrin palmitoylation, either downstream or upstream of phosphorylation events, might anchor it to the PSD and help recruit GABAergic synapse-specific molecules such as NLGN2 and CB. Furthermore, the identification of gephyrin acetylation at lysine and Ser/Thr residues[133,181] suggests that this modification might have an important role in determining its phosphorylation status[182] and, possibly, its synaptic function. This possibility is reinforced by the existence of residues that can be modified by both phosphorylation and acetylation.

SUMOylation is another posttranslational modification that occurs at lysine residues, exerting a plethora of effects on target proteins, notably the modulation of protein–protein

interactions and scaffolding functions (reviewed in Refs [183,184]), as well as neurotransmitter receptor trafficking and function[185,186]. Our own studies, using *in vitro* assays, have shown that gephyrin is a SUMO substrate, and its postsynaptic clustering properties are altered upon overexpression of SUMOylating and de-SUMOylating enzymes in primary neuronal cultures (Tyagarajan et al., 2012, Neuroscience Meeting Planner. New Orleans, LA, Program number: 745.12.). Considering that gephyrin contains several surface-exposed lysine residues, various post-translational modifications involving multiple signal transduction pathways are likely to modulate its postsynaptic functions and aggregation properties.

Finally, susceptibility to calpain, a Ca^{2+}-dependent protease, seems to be a downstream consequence of gephyrin post-translational modification[125,133]. Graded gephyrin proteolysis provides a mechanism for activity-dependent, dynamic regulation of gephyrin scaffolds and, hence, GABAergic transmission[173]. Ca^{2+}-independent activation of calpain can be mediated by neurotrophins[187], and calpain targeting of the K^+/Cl^- co-transporter KCC2 has been recently reported[188], indicating that multiple modes of activity-dependent regulation of neuronal networks and gephyrin availability exist.

BOX 3 Activity-dependent transcription and translation of gephyrin-interacting molecules

Neuronal activity triggers, notably via Ca^{2+} mobilization, specific signal transduction pathways that culminate into nuclear transcriptional regulation (reviewed by Ref. [189]). Notably, recent studies have identified activity-dependent translocation of specific transcription factors and proteins from the synapse to the nucleus[190,191]. Furthermore, neuronal activity-dependent epigenetic changes in histone modifications and DNA methylation patterns have an important role in modulating synaptic plasticity. For instance, using an improved genome-wide profile analysis to investigate the DNA methylome, changes in the DNA methylation states implicate DNA modification as a general mechanism for activity-dependent nuclear changes[192].

Non-coding RNAs have also emerged as important modulators of neuronal development, structure and function (reviewed by Ref. [193]). Hence, any alteration in the transcriptional profile of a neuron is bound to affect its function outside the nucleus, including the synapse. Furthermore, neuronal activity not only shapes gene regulation at the transcriptional level, but also influences alternative splice site selection[194], dendritic transport of mRNA[195] and local protein synthesis (reviewed by Ref [196]). Novel candidates for synapse regulation have been identified using RNA interference screens[197,198]. One such screen revealed the importance for the transcription factor NPAS4 in modulating the expression of other transcription factors regulating GABAergic synapse formation in principal cells[161]. In another screen, the transcription factor myocyte enhancer factor 2 (MEF2) was identified as an important regulator of activity-dependent transcriptional change[199]. The importance of MEF2 for dendritic differentiation and synapse formation had already been recognized[183]; however, in this study, the authors found activity-dependent PolyA site selection in transcribed mRNAs, leading to changes in protein production that subsequently affected synaptic plasticity. One of the target transcript affected by the choice of PolyA site selection is *Arhgef9* mRNA (which encodes CB), suggesting that MEF2 regulates both excitatory and inhibitory synapses.

Given the importance of gephyrin for inhibitory neurotransmission, its transcriptional and post-transcriptional regulation has received little attention. However, analysis of the murine gephyrin promoter uncovered the presence of multiple SP1 sequences in the 5'UTR sequence, favouring widespread transcription of gephyrin in multiple cell types[200]. There is little evidence to suggest that *Gphn* transcription and mRNA splicing is modulated by neuronal activity (but see Ref [153]). However, a new technique, referred to as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), identified the neuron-specific splicing factor NOVA as a regulator of gephyrin mRNA splicing in mouse neurons[201]. In principle, alternative splicing of the gephyrin transcript might also act as an additional mechanism for dynamic adaptation of neuronal function, where production of specific splice variants might modify the signalling properties at specific synapses. A similar activity-dependent splicing mechanism has been reported for neurexin-1 (*Nxr1*) mRNA exon selection by the RNA binding protein SAM68 [194]. Previous work on SAM68 has demonstrated its association with polyadenylated mRNA in cortical and hippocampal lysates;

although gephyrin mRNA was not found to be SAM68-associated, it is possible that other GABAergic synapse-specific molecules, such as CB or NLGN2, are substrates for SAM68-dependent splicing.

Over the past decade, local protein synthesis has been shown to be essential for various forms of long-lasting synaptic plasticity. However, only a few mRNAs have been reported to be synaptically localized and translated. Recently, analysis of the synaptic neuropil using deep sequencing technology identified 2,550 different transcripts localized at dendrites and/or axons[195], including a sizeable fraction of inhibitory synapse-specific mRNAs and signalling molecules, providing a basis for future analysis. Although activity-dependent transcription, splicing, mRNA dendritic transport and local protein translation at inhibitory synapses have largely remained unexplored to date, the results of the study above emphasize that these mechanisms might also have an essential role in modulating GABAergic synapse formation and plasticity.

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Figure legends

Figure 1

Postsynaptic localization and clustering of gephyrin in neurons. A-C: Double-immunofluorescence staining for gephyrin (green) and vesicular GABA transporter (vGAT, magenta; a marker of inhibitory presynaptic terminals) in the CA1 region (stratum radiatum) of an adult mouse, depicting in various color combinations that the vast majority of gephyrin clusters are apposed to a presynaptic terminal, and thus are located postsynaptically (scale bar, 15 μm; for details about the methods, see Ref [125]). D: Electron micrograph depicting gephyrin immunogold labelling (black dots) in a reciprocal synapse between an olfactory bulb granule cell (GC) spine and a mitral cell dendrite (MC); the thick arrow points to the symmetric (GABAergic) PSD, containing the gephyrin immunolabelling close to the postsynaptic membrane, whereas the thin arrow points to the asymmetric (glutamatergic) PSD (scale bar, 100 nm; for details about the methods, see Ref [202]). E-G: Post-synaptic clustering of eGFP-gephyrin transfected in a primary rat hippocampal neurons at 8 days-in-vitro, and visualized 7 days later, along with immunofluorescence for endogenous gephyrin (red) and synapsin 1 (blue; a marker of presynaptic terminals). The arrow points to the axon-initial segment, in which the antibody against gephyrin produces no labelling, reflecting a phosphorylation-specific gephyrin conformation, not detected by this antibody[126]. To underscore the apposition between gephyrin clusters and presynaptic terminals, VGAT staining is shown in addition to GFP-gephyrin in the enlargement of boxed area in E (scale bar, 20 μm; for details about the methods, see Ref [125]).

Figure 2

Schematic depictions of the molecular heterogeneity of inhibitory PSDs and regulation of gephyrin clustering in GABAergic synapses. A) In glycinergic synapses, GlyRs cluster with the gephyrin scaffold, which in turn is linked to the actin (and presumably tubulin) cytoskeleton (see Supplementary Table 1). Heat Shock Cognate 71kDa Protein (HSC70) negatively regulates gephyrin clustering. A putative role for a GEF anchored in the PtdIns(3,4,5)P_3 in the plasma membrane is indicated by a question mark. The type of NLGN present in these synapses includes NLGN3 and NLGN4. B) 'Prototypical' GABAergic synapses contain several additional molecules not present in glycinergic synapses. These include CB isoforms, which contribute to gephyrin clustering by interacting with NLGN2, $GABA_A$Rs, gephyrin and CDC42. CB is anchored to PtdIns(3,4,5)P_3 in the plasma membrane

via its PH domain. GRIP1 binding to gephyrin might serve to anchor nNOS to the gephyrin clusters via a PDZ domain-mediated interaction. C) Some GABAergic synapses, such as those found in cerebellar Purkinje cells and perisomatically in cortical pyramidal cells, associate with the DGC (comprising α- and β-dystroglycan, dystrophin, dystrobrevins, syntrophins). CB is not depicted here, as its presence in these synapses has never been investigated. Various signalling molecules are known to be associated with the DGC, including nNOS and PIN/DLC-1. The DGC is linked indirectly to NLGN2 via S-SCAM, which also interacts with SynArfGEF, an activator of Arf6. Actin microfilaments, to which dystrophin binds, are depicted. D) Gephyrin clustering at GABAergic synapses is modulated by various posttranslational modifications. GSK3β and ERK1/2, which target Ser270 and Ser268, respectively, cooperate with calpain to negatively modulate gephyrin clustering. The scheme also indicates the need for Ca^{2+} (from unknown sources) for activating calpain (and thereby cleave gephyrin molecules), as well as activating Ca^{2+}-activated protein kinases (and phosphatases), such as CaMKII for example, which are known to regulate $GABA_AR$ phosphorylation. In addition, MDGA interacts with NLGN2 to suppress the formation of GABAergic synapses, whereas IgSF9b (which is enriched in cortical interneurons) interacts with NLGN2 via S-SCAM to facilitate GABAergic synapse formation.

Figure 3

Role of gephyrin as a master regulator of neuronal function. The scheme depicts the hypothesis that gephyrin is a central component of a signalling hub contributing to homeostatic synaptic plasticity. According to this model (right), adaptations of gephyrin clustering properties contribute to modulate GABAergic and glutamatergic synaptic function to help maintaining excitatory-inhibitory balance. The specific mechanisms involved are depicted on the left. Thus, changes in network activity, as well as various extracellular signals, lead to increased Ca^{2+} signalling and activation of effector molecules that modulate gephyrin clustering properties and its interactions with proteins of the PSD, as well as $GABA_AR$ functions. These structural and functional adaptations affect GABAergic synaptic plasticity and signalling pathways downstream of GABAergic synapses, thereby contributing to adjust network activity and extracellular signals. The scheme on the left highlights the role of intracellular Ca^{2+} influx for activating proteins kinases and phosphatases, as well as the protease calpain. By acting on gephyrin, these effectors alter its scaffolding properties, resulting in changes in gephyrin cluster size and density. In addition, posttranslational modifications of $GABA_ARs$ contribute to adjust their functional properties, in coordination with their anchoring to the gephyrin scaffold. The resulting net changes determine the

strength of GABAergic inhibition, which in turn, adjusts the excitability of the neuron and the network. Effector molecules interacting with gephyrin can induce changes in down-stream signalling events, leading to structural and functional reorganization.

Glossary

Cys-loop ligand-gated ion channels: Sub-family of neurotransmitter receptors, comprising the prototypic nicotinic acetyl choline receptor, $GABA_A$ and GlyR, and $5HT_3$-receptors, which form a pentameric homo- or heteromeric integral ion channel mediating fast synaptic transmission.

Dystroglycan: Protein encoded by the DAG1 gene, encoding two exons translated into two non-covalently bound protein products, α- and β-dystroglycan, which are key constituents of the dystrophin-glycoprotein complex (DGC).

Inhibitory neurotransmission: Effect of neurotransmitters, notably GABA and glycine, which lower the resting membrane potential, increase membrane resistance (also called "shunting inhibition") and reduce excitability of neurons, e.g. by activating voltage-gated K^+ or Ca^{2+} channels.

Post-synaptic density: Generic term derived from the ultrastructural appearance of the post-synaptic membrane, which is thicker and more electron-dense than the plasma membrane, due to accumulation of molecules (e.g., transsynaptic extracellular matrix proteins, neurotransmitter receptors, and effector proteins) held together by scaffolding proteins and binding to the actin cytoskeleton.

Protein post-translational modification (PTM): structural change (e.g., formation of disulphide bonds between two amino acids), or reversible attachment of functional residues (e.g., a phosphate or acetate group), a small protein (e.g., ubiquitin, SUMO), or a lipid (e.g., palmitic acid) to specific residues of a protein, mediated by a dedicated enzyme or enzymatic pathway and conferring novel properties to the modified substrate (e.g., functional activation, membrane-anchorage, targeting for trafficking or degradation).

Tonic inhibition: Long-lasting, low amplitude, form of inhibitory neurotransmission mediated by extrasynaptic $GABA_AR$ activated by ambient GABA in the extracellular fluid.

Protein scaffold: High-order molecular arrangement of proteins, forming a highly structured, crystal-like lattice by self-assembly or via specific protein interaction motives and serving to anchor other proteins (notably neurotransmitter receptors and effector molecules) at specific subcellular sites.

Supplementary data

Table 1 Gephyrin-interacting molecules

Full name	Description	Role of interaction	Ref.
Calpain	Ca^{++}- and ERK-dependent serine proteases enriched in the CNS and with a broad substrate specificity	Cleaves gephyrin in a manner regulated by phosphorylation of Ser268 and Ser270	1
Cyclin-dependent kinase 5 (CDK5)	Ser/Thr kinase	Phosphorylates gephyrin residue Ser270 in a collybistin-dependent manner	2
Collybistin*	Guanine exchange factor; member of the Dbl family	Essential for gephyrin clustering in most GABAergic synapses; selectively activates CDC42	3-7
Cell division control protein 42 homolog (CDC42)	Small GTPase of the Rho family	Modulates gephyrin postsynaptic cluster size and shape, in conjunction with CB	8,9
Dynein light chain 1 and 2 – Protein inhibitor of neuronal nitric oxide synthase (PIN)*	Accessory proteins of the dynein motor complex; protein inhibitor of nNOS	Their role in gephyrin transport is not clear; PIN might regulate nNOS activity at GABAergic synapses	10,11
Mitogen activated protein kinase 1 and 3 (ERK 1/2)	Ser/Thr kinases; members of the MAP kinase family	Phosphorylation of gephyrin Ser268 to regulate postsynaptic gephyrin cluster size	12
$GABA_A$ receptor α1, α2, α3, β2, β3 subunits*	Subunits contributing to $GABA_AR$ subtypes mediating phasic inhibition	Involved in region specific localization of specific $GABA_AR$ subunits in neurons and initiating gephyrin clustering at synapses	13-18
$GABA_A$ Receptor Associated Protein (GABARAP)*	Member of the autophagy-related protein 8 family; sequence homology with MAP1 LC3	Initially it was thought to link gephyrin with $GABA_AR$ subunit (γ2); however, it facilitates cell surface expression of GABAAR and regulates anterograde transport along with KIF5	19-21
Glycine receptor β subunit*	GlyR subunit contributing to heteromeric receptors	High affinity binding site, required for postsynaptic clustering of heteromeric GlyR	22,23
Glutamate Receptor Interacting Proteins (GRIP)	PDZ-domain containing proteins located in glutamatergic and GABAergic PSDs, interacting with numerous proteins involved in trafficking and localization of synaptic proteins	Function of GRIP at GABAergic synapses is presently unclear.	24,25
Glycogen Synthase Kinase 3β (GSK3β)	Proline-directed Ser/Thr kinase involved in energy metabolism, neuronal cell development, and body	Targets Ser270 of gephyrin to regulate formation of postsynaptic clusters and density of GABAergic synapses	1,12

	pattern formation		
Heat Shock Cognate 71kDa Protein (HSC70)	Chaperone; repressor of transcriptional activation	Direct interaction with gephyrin (G-domain); overexpression interferes with gephyrin, but not GlyR clustering	26
Integrins β1 and β3	Membrane proteins connecting extracellular matrix (ECM) to intracellular signaling. They play an important role in LTP and spatial memory.	β1 and β3 integrins signal in opposite directions to regulate gephyrin and GlyR clustering in spinal cord neurons in a CaMKII, thrombospondin 1 and fibrinogen dependent mechanism.	27,28
KIF5	Accessory protein of kinesin-based motor complexes	Contributes to anterograde transport of gephyrin/GlyR complexes, as well as $GABA_AR$/HAP1 and $GABA_AR$/ GABARAP/gephyrin complexes	20,29,30
Mena/Vasp*	Proteins regulating actin polymerization	Postulated to contribute to gephyrin binding to microfilaments	31,32
Mammalian Target of Rapamycin (mTOR)	serine/threonine protein kinase regulating cell growth, proliferation, survival, motility, protein synthesis, and transcription	Unknown; gephyrin postulated to regulate mTOR activity	33,34
Neuroligin2 (NLGN2)*	Member of the neuroligin family, preferentially located at GABAergic synapses	NL2 binding to gephyrin might contribute to initial formation of postsynaptic clusters; contributes to postsynaptic aggregation of α1-$GABA_AR$ in the absence of gephyrin	35-37
Peptidyl-prolyl cis/trans isomerase 1 (Pin1)*	Isomerizes serine and threonine residues post-phosphorylation	Postulated to regulate gephyrin conformation and interaction with GlyR in a phosphorylation-dependent manner	38
Protein phosphatase 1	Serine/threonine phosphatase with a 30kDa catalytic subunit that can interact with diverse regulatory subunits	Regulates gephyrin clustering; targeted residues not identified.	39
Profilin	Regulates actin dynamics and turnover; binds proline-rich domains, and phosphoinositides in the plasma membrane	Postulated to contribute to anchoring gephyrin to actin microfilaments	32,40
Vacuolar sorting protein 35 /Neurobeachin	Contribute to protein sorting and transport, and to synapse delivery of neurotransmitter receptors	Postulated to contribute to gephyrin and GlyR trafficking to the synapse	41
WAVE-associated Rac GTPase Activating Protein (WRP)/SLIT-ROBO Rho GTPase-activating protein	Homologous small GTPases of the Rho family	Identified in a screen of SH3 domain interacting proteins; facilitates gephyrin and $GABA_AR$ postsynaptic clustering	42

2 (srGAP2)			

* indicates that the localization of the binding site on gephyrin is known

Supplementary Figure 1

The three domains of gephyrin are indicated by their name (G, C, E). Top and middle: sites identified in mouse gephyrin containing the C3 and C4A splice cassettes; no phospho-residues were identified within these two cassettes, suggesting that phosphorylation regulates functions common to most gephyrin isoforms. The arrows indicate where the cassettes are incorporated in the C domain Bottom: sites identified in rat P1 gephyrin; the color code identifies the study reporting the respective sites. Symbols indicate identical sites in the various splice isoforms (with numbering shifted due to splice cassette insertion at the dotted line). Ac, acetylation; Ac/P, acetylation and phosphorylation.

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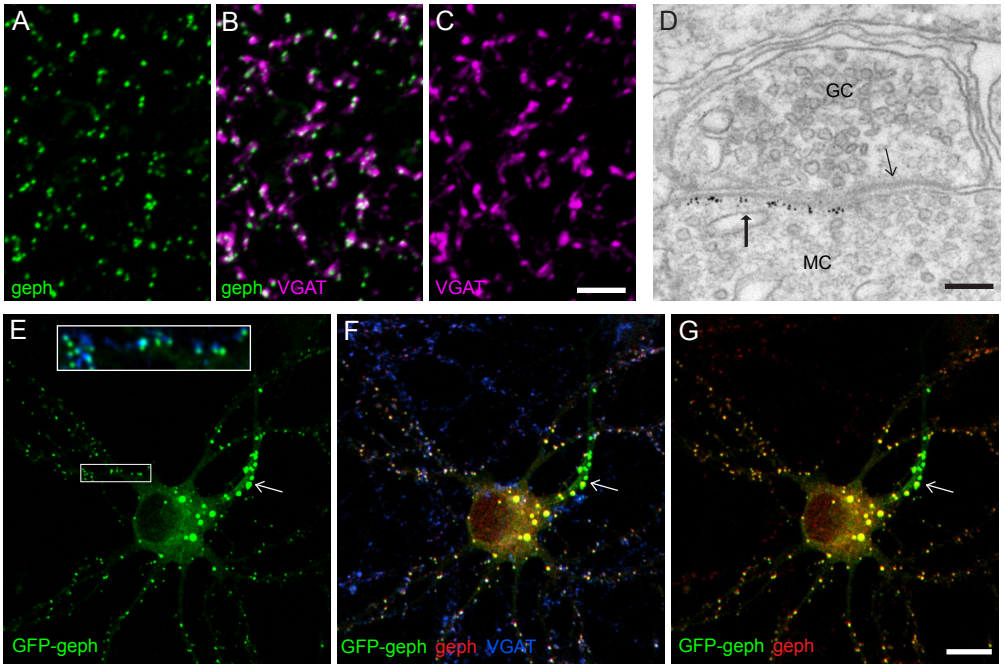
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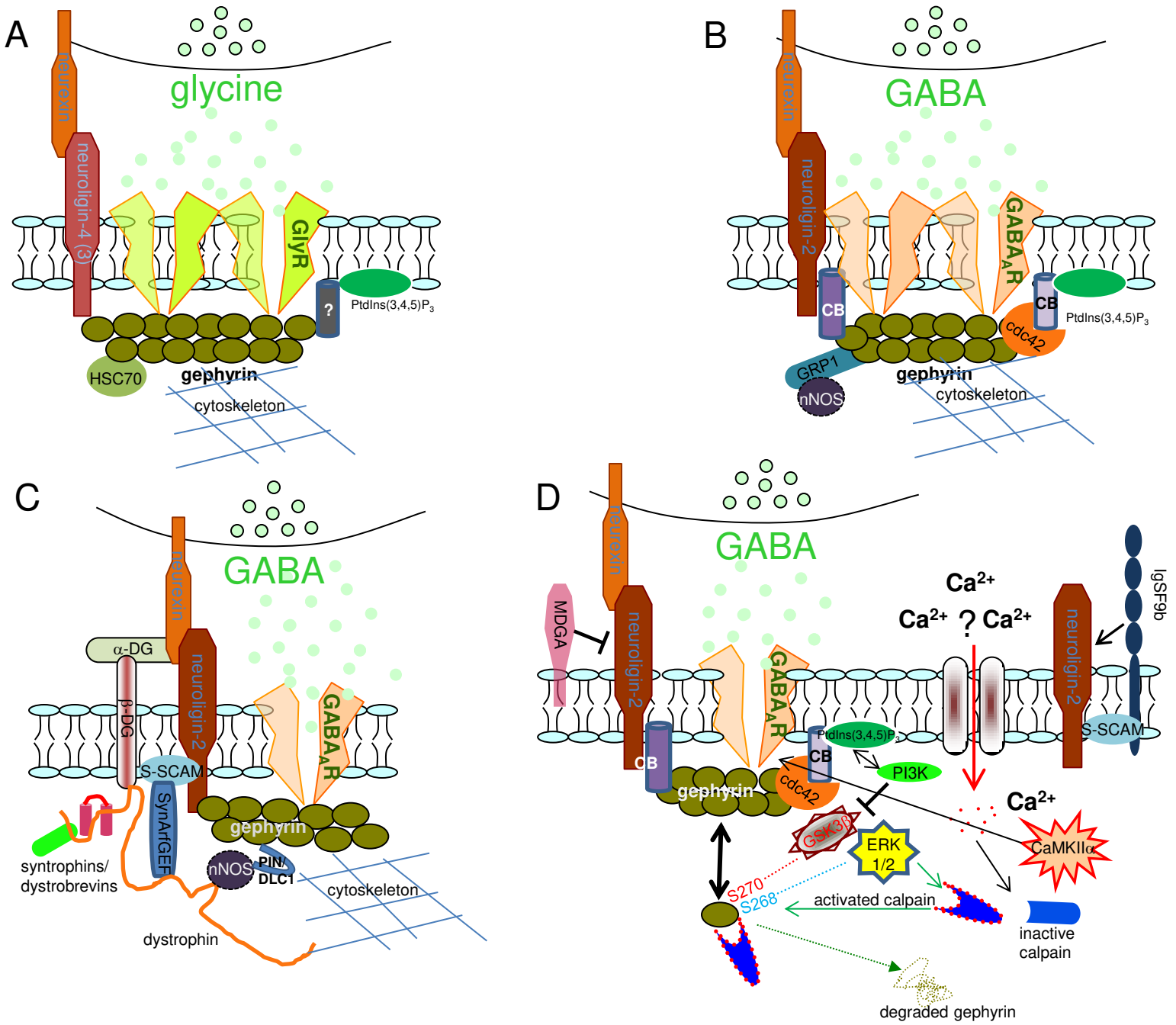
Figure 1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α-DG
β-DG
neuroligin-2
GABAAR
S-SCAM
SynArfGEF
gephyrin
syntrophins/
dystrobrevins
nNOS
PIN
DLC1
cytoskeleton
dystrophin
D
GABA
neurexin
MDGA
neuroligin-2
GABAAR
Ca2+
Ca2+ ? Ca2+
neuroligin-2
IgSF9b
S-SCAM
PtdIns(3,4,5)P3
CB
CB
PI3K
cdc42
gephyrin
Ca2+
GSK3β
ERK
1/2
CaMKIIα
S270
S268
activated calpain
inactive
calpain
degraded gephyrin

Figure 2

Gephyrin as a hub for synaptic homeostasis

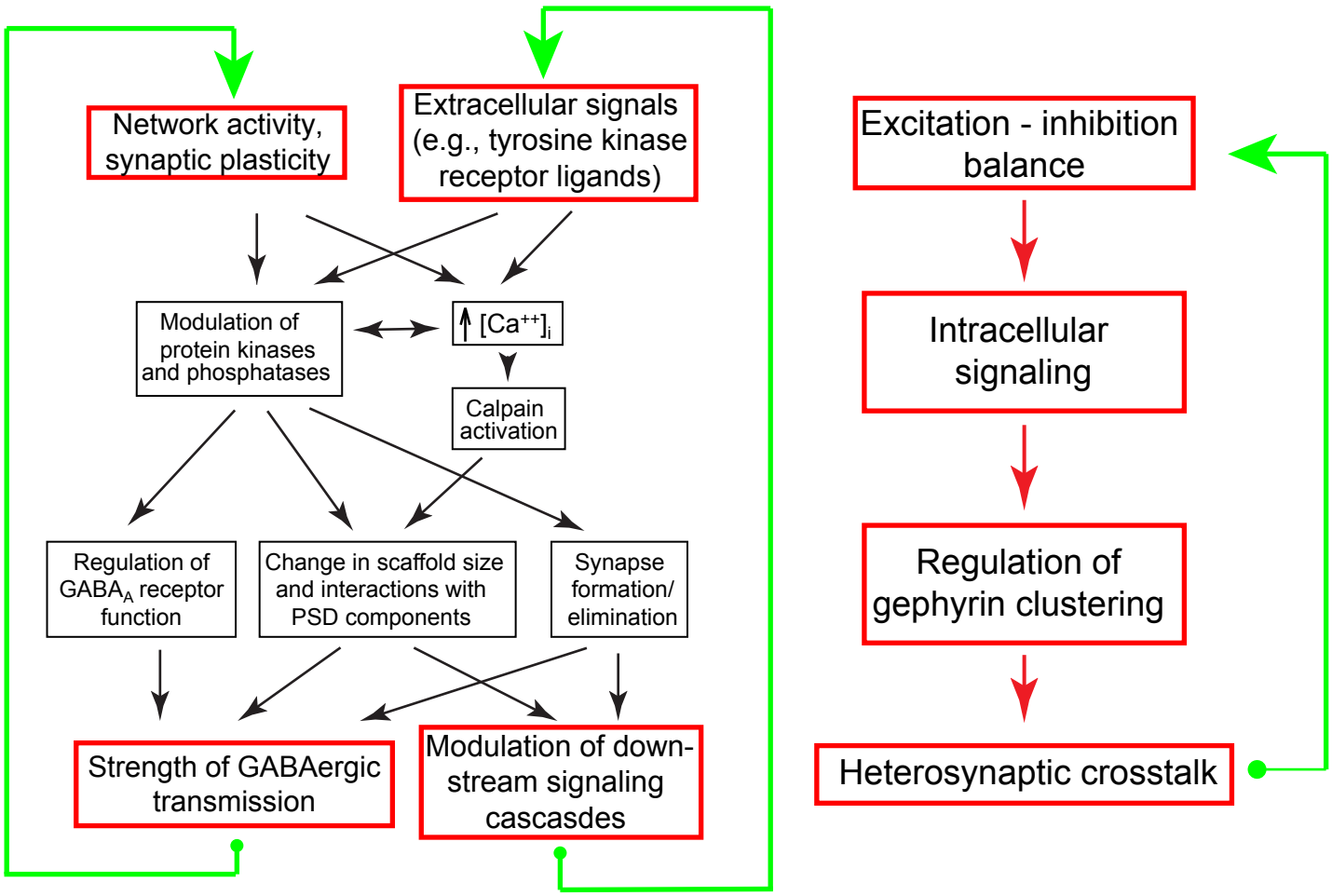
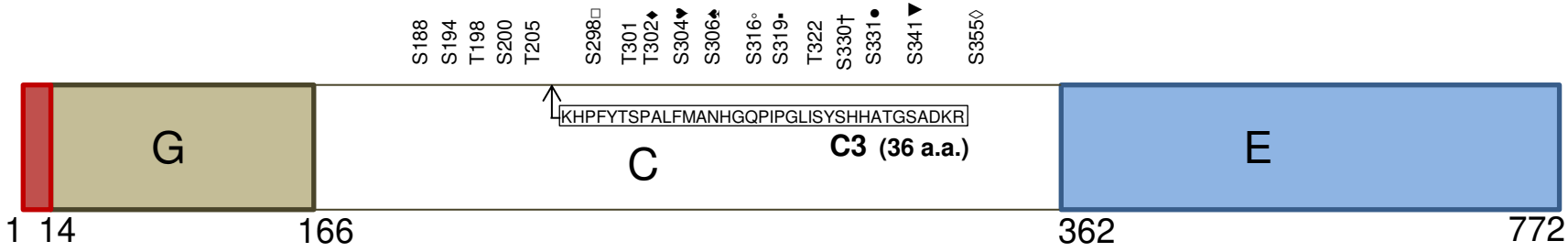
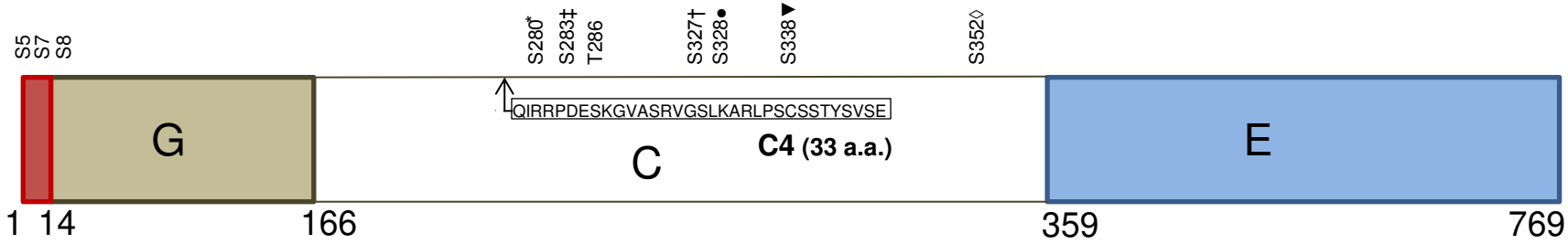


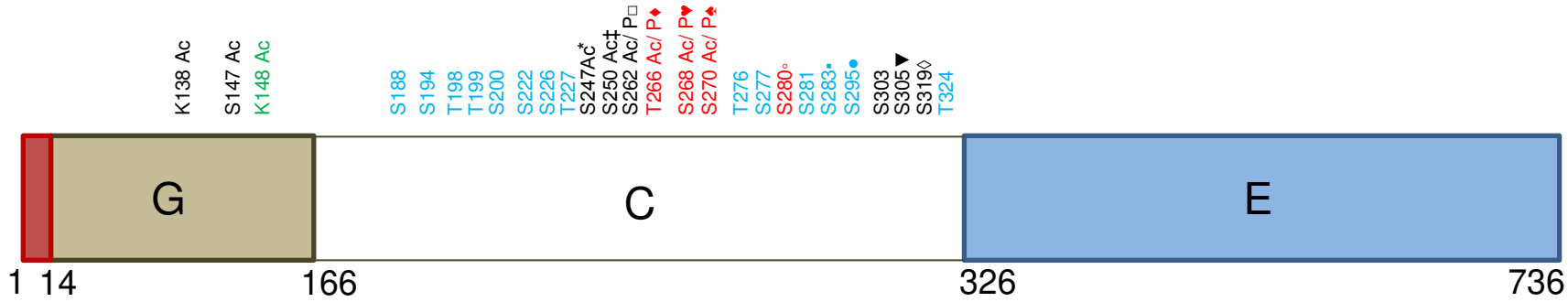
Figure 3



Huttlin et al., Cell 2010



Huttlin et al., Cell 2010



Tyagarajan et al., JBC 2013
Herwig and Schwarz, JBC 2012
Tyagarajan et al., JBC 2013 and Herwig and Schwarz, JBC 2012
Schwer et al., Aging Cell 2009

Suppl. Figure 1